Hypoxia enhances the regenerative effects of Erythropoietin and its non-erythropoietic peptide analogue in models of Endothelial Cell Injury

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Background: Hypoxia is invariably associated with wound repair, inflammation, and vascular disease. The induction of Hypoxia Inducible Factor-1 (HIF-1) is a characteristic feature of hypoxia, and orchestrates the profound changes in transcription that accompany hypoxia. HIF-1 expression is localized to several cell types, and regulates several genes that are important to vascular function including vascular endothelial growth factor (VEGF), nitric oxide synthase (NOS), endothelin-1 and erythropoietin (EPO). In fact, EPO derived from vascular endothelial cells appears to be important in protecting the endothelium against ischemic injury. The non-erythropietic analogue of EPO; pyroglutamate helix B surface peptide (pHBSP) retains the protective actions of EPO without its erythropoietic effects. The aim of our study was to assess the reparative effects of these molecules when used in combination with HIF inducers.

Method: The reparative effects of EPO and pHBSP were assessed under hypoxia (1% O_2) and normoxia (21% O_2) as well as in the presence or absence of DMOG; a HIF-1 inducer. An *in vitro* model of wound healing (the scratch assay) was used: a monolayer of rat aortic endothelial cells (RAECs) was scraped to produce a reproducible injury, and the scratch closure was assessed over 24 h. An *in vivo* model of vascular injury using a 2F fogarty balloon catheter was introduced into the common carotid artery causing complete removal of the vascular endothelium. Drugs were applied locally onto the injured arteries using a hydrogel (30% w/v) and re-endothelialisation assessed using Evans blue staining injected 30 min intravenously before culling the rat. The effects of EPO and pHBSP on cell proliferation, chemotaxis and apoptosis were assessed in both the *in vitro* and *in vivo* models. The potential molecular mechanisms of these effects were also explored.

Results: *In vitro,* EPO and its analogues only exhibited a reparative effect under hypoxic conditions (13 ± 2.6 %, and 10 ± 1.69 %, $p< 0.01$ improvement in the degree of endothelial cell closure after treatment with EPO and pHBSP respectively) compared to normoxic conditions (3.2 ± 0.9%, $p>0.05$). These effects appeared to be mediated by promoting RAEC proliferation and migration of ($p<0.05$). The priming effect of hypoxia was associated with stabilization of HIF-1α. Hypoxia was associated with a reduction in nitric oxide (NO) production as assessed by its oxidation products nitrite and nitrate, and this was consistent with the oxygen requirement for the endogenous production of NO by NO synthase (NOS).The HIF-1 inducer; DMOG also exhibited reparative effects in a concentration dependent manner. Similar results were observed *in vivo* where DMOG and EPO accelerated the repair of injured arteries (35 ± 9.8 % recovery compared to untreated injured arteries respectively). This mode of application also caused site-specific increase in VEGF expression on treated arteries compared to untreated ones within the same animal.

Conclusion and implication: The tissue-protective effects of EPO-related cytokines in pathophysiological settings are enhanced by hypoxia. These findings may be particularly relevant to atherogenesis and post-angioplasty restenosis